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[ITEM] SPECIFICATION 1
[ITEM] DRAWINGS 1
[ITEM] ABSTRACT 1

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[Title of the Invention] Dipeptide Producing Method, L-Amino Acid Amide Hydrolase Used Therein, and L-Amino Acid Amide Hydrolase Producing Method

[Scope of Claims for Patent]

[Claim 1] A method for producing a dipeptide from an L-amino acid amide and an L-amino acid using a culture of a microbe having the ability to produce a dipeptide from an L-amino acid amide and an L-amino acid, microbial cells isolated from the culture, or a treated microbial product of the microbe.

[Claim 2] The method for producing a dipeptide according to claim 1, wherein the microbe belongs to the genus *Bacillus, Corynebacterium, Erwinia, Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodosporidium, Sporobolomyces, Tremella, Torulaspora, Sterigmatomyces,* or *Rhodotorula*.

[Claim 3] The method for producing a dipeptide according to claim 1 or 2, wherein the L-amino acid amide is L-alanine amide.

[Claim 4] The method for producing a dipeptide according to any one of claims 1 to 3, wherein the L-amino acid is L-glutamine or L-asparagine.

[Claim 5] An L-amino acid amide hydrolase obtained from a microbe belonging to the genus *Bacillus, Corynebacterium, Erwinia, Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodosporidium, Sporobolomyces,*

Tremella, Torulaspora, Sterigmatomyces, or Rhodotorula, which catalyzes a reaction that produces a dipeptide from an L-amino acid amide and an L-amino acid.

[Claim 6] A method for producing an L-amino acid amide hydrolase comprising: culturing a microbe belonging to the genus Bacillus, Corynebacterium, Erwinia, Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodosporidium, Sporobolomyces, Tremella, Torulaspora, Sterigmatomyces, or Rhodotorula in a medium, and accumulating in the medium and/or cells an L-amino acid amide hydrolase that catalyzes a reaction that produces a dipeptide from an L-amino acid amide and an L-amino acid.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to a method for producing a dipeptide both conveniently and inexpensively without going through a complex synthesis method, and more particularly, to a method for producing a dipeptide from an L-amino acid amide and an L-amino acid, an L-amino acid amide hydrolase used in the dipeptide producing method, and a method for producing the L-amino acid amide hydrolase.

[0002]

[Prior Art]

Dipeptides are used in the field of pharmaceutical materials and functional foods and various fields. For example, L-alanyl-L-glutamine

is used as a component of serum-free media, and is used for infusion components since it has greater stability and higher solubility than L-glutamine.

[0003]

Chemical synthesis methods, which have been conventionally known as methods of producing dipeptides, are not necessarily simple. Known examples of such methods include a method that uses N-benzyloxycarbonylalanine (hereinafter, "Z-alanine") and protected L-glutamine (see Bull. Chem. Soc. Jpn., 34, 739 (1961), Bull. Chem. Soc. Jpn., 35, 1966 (1962)), a method that uses Z-alanine and protected L-glutamate-γ-methyl ester (see Bull. Chem. Soc. Jpn., 37, 200 (1964)), a method that uses a Z-alanine ester and unprotected glutamic acid (see Japanese Patent Application Laid-Open Publication No. H1-96194), and a method that uses a 2-substituted-propionyl halide as raw material and synthesizes an N-(2-substituted)-propionyl glutamine derivative as an intermediate (see Japanese Patent Application Laid-Open Publication No. H6-234715).

[0004]

However, in all of these methods, the introduction and elimination of a protecting group or the synthesis of an intermediate is required, so that these production methods have not been sufficiently satisfactory in view of their industrial advantages.

[0005]

In addition, known examples of methods for producing a dipeptide using a microbial enzyme system include a method (Japanese

Patent Application Laid-Open No. S53-92729) that uses methyl ester of Z-aspartic acid and phenylalanine, and a method (Japanese Patent Application Laid-Open No. H10-136992) that uses an aspartic acid amide and a methyl ester of phenylalanine. Other known examples of methods for producing a dipeptide using an enzymatic process include the methods described in EPA 0278787 and WO 90/01555.

[0006]

However, since it is necessary to use an amino acid methyl ester as the starting material in each of the microbial enzyme systems, there is a demand to develop a method for producing dipeptides that uses raw material which can be acquired comparatively inexpensively, in an industrially advantageous and simple pathway.

[0007]

[Problems to be Solved by the Invention]

An object of the present invention is to provide a method for producing dipeptides that uses starting materials which can be acquired comparatively inexpensively, in an industrially advantageous and simple pathway.

[0008]

[Means to Solve the Problems]

As a result of conducting extensive research in consideration of the aforementioned object, the inventors of the present invention have found that certain types of microbes have the ability to produce dipeptides which can be acquired comparatively inexpensively from L-amino acid amides and L-amino acids, thereby leading to completion

of the present invention.

[0009]

The present invention provides as follows.

[0010]

(Claim 1) A method for producing a dipeptide from an L-amino acid amide and an L-amino acid using a culture of a microbe having the ability to produce a dipeptide from an L-amino acid amide and an L-amino acid, microbial cells isolated from the culture, or a treated microbial product of the microbe.

[0011]

(Claim 2) The method for producing a dipeptide according to claim 1, wherein the microbe belongs to the genus *Bacillus, Corynebacterium, Erwinia, Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodosporidium, Sporobolomyces, Tremella, Torulaspora, Sterigmatomyces,* or *Rhodotorula*.

[0012]

(Claim 3) The method for producing a dipeptide according to claim 1 or 2, wherein the L-amino acid amide is L-alanine amide.

[0013]

(Claim 4) The method for producing a dipeptide according to any one of claims 1 to 3, wherein the L-amino acid is L-glutamine or L-asparagine.

[0014]

(Claim 5) An L-amino acid amide hydrolase obtained from a microbe belonging to the genus *Bacillus, Corynebacterium, Erwinia,*

Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodosporidium, Sporobolomyces, Tremella, Torulaspora, Sterigmatomyces, or Rhodotorula, which catalyzes a reaction that produces a dipeptide from an L-amino acid amide and an L-amino acid.

[0015]

(Claim 6) A method for producing an L-amino acid amide hydrolase comprising: culturing a microbe belonging to the genus Bacillus, Corynebacterium, Erwinia, Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodosporidium, Sporobolomyces, Tremella, Torulaspora, Sterigmatomyces, or Rhodotorula in a medium, and accumulating in the medium and/or cells an L-amino acid amide hydrolase that catalyzes a reaction that produces a dipeptide from an L-amino acid amide and an L-amino acid.

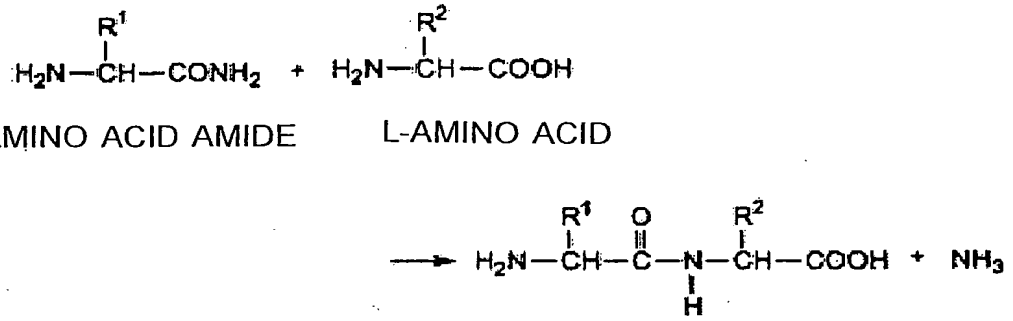
[0016]

[Embodiments of the Invention]

The method for producing a dipeptide of the present invention uses a culture of a microbe having the ability to produce a dipeptide from an L-amino acid amide and an L-amino acid, microbial cells isolated from the culture, or a treated microbial product of the microbe. The reaction in the method for producing a dipeptide of the present invention is represented by the following reaction formula:

[0017]

[Chemical 1]



L-AMINO ACID AMIDE L-AMINO ACID

(wherein R^1 represents an amino acid side chain of an L-amino acid amide, and R^2 represents an amino acid side chain of an L-amino acid).

[0018]

Amino acid amides are compounds which can be acquired comparatively inexpensively as commercially available products. The method of the present invention that uses as starting materials an amino acid amide and an unprotected amino acid is a completely new method for producing dipeptides not found in the prior art, and makes it possible to provide dipeptides useful as pharmaceutical materials and functional foods more economically.

[0019]

Hereinafter, the dipeptide producing method of the present invention will be described in detail with reference to the attached drawings in the following order:

[I] Microbes Having the Ability to Produce Dipeptides from L-amino Acid

Amides and L-Amino Acids

[II] Properties of L-Amino Acid Amide Hydrolases

[III] Dipeptide Producing Method.

[0020]

[I] Microbes Having the Ability to Produce Dipeptides from L-Amino Acid Amides and L-Amino Acids

Microbes having the ability to produce dipeptides from L-amino acid amides and L-amino acids can be used without any particular restrictions as the microbe used in the present invention. Examples of microbes having the ability to produce dipeptides from L-amino acid amides and L-amino acids include microbes belonging to the genus *Bacillus, Corynebacterium, Erwinia, Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodosporidium, Sporobolomyces, Tremella, Torulaspora, Sterigmatomyces,* and *Rhodotorula*, and specific examples thereof are indicated below.

[0021]

Bacillus megateirum AJ3284	FERM P-18421
Corynebacterium glutamicum	ATCC 13286
Erwinia carotovora AJ2719	FERM P-18420
Rhodococcus rhodochrous	ATCC 19149
Chryseobacterium meningosepticum	ATCC 13253
Micrococcus luteus	ATCC 9341
Pseudomonas saccharophilia	ATCC 15946
Cryptococcus albidus var. albidus	IFO 0378

Trichosporon gracile	ATCC 24660
Rhodosporidium diobovatum	ATCC 22264
Sporobolomyces salmonicolor	IFO 1038
Tremela foliacea	IFO 9297
Torulaspora delbrueckii	IFO 1083
Sterigmatomyces elviae	IFO 1843
Rhodotorula ingeniosa	ATCC 22993

[0022]

Bacillus megateirum strain AJ3284 is a microbe that was deposited at the independent administrative corporation, International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology on July 13, 2001, and was assigned the deposit number of FERM-P18421. Further, *Erwinia carotovora* strain AJ2719 is a microbe that was deposited at the independent administrative corporation, International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology on July 13, 2001, and was assigned the deposit number of FERM-P18420.

[0023]

Wild strains or variant strains may be used for these microbes, and recombinant strains and so forth derived by cell fusion, genetic manipulation or other genetic techniques may also be used.

[0024]

To obtain microbial cells of these microbes, the microbes can be cultured and grown in a suitable medium. There is no particular restriction on the medium used for this purpose so far as it allows the

microbes to grow. This medium may be an ordinary medium containing ordinary carbon sources, nitrogen sources, phosphorous sources, sulfur sources, inorganic ions, and organic nutrient sources as necessary.

[0025]

For example, any carbon source may be used so far as it can be utilized by the aforementioned microbes, and specific examples of which that can be used include sugars such as glucose, fructose, maltose, and amylose, alcohols such as sorbitol, ethanol and glycerol, organic acids such as fumaric acid, citric acid, acetic acid and propionic acid and their salts, hydrocarbons such as paraffin as well as mixtures thereof.

[0026]

Examples of nitrogen sources that can be used include ammonium salts of inorganic acids such as ammonium sulfate and ammonium chloride, ammonium salts of organic acids such as ammonium fumarate and ammonium citrate, nitrates such as sodium nitrate and potassium nitrate, organic nitrogen compounds such as peptones, yeast extract, meat extract and corn steep liquor as well as mixtures thereof.

[0027]

In addition, nutrient sources used in ordinary media, such as inorganic salts, trace metals and vitamins, can also be suitably mixed and used.

[0028]

Microbial cells may be acquired that have a high level of activity to produce dipeptides from L-amino acid amides and L-amino acids by further adding L-amino acid amide to the medium.

[0029]

There is no particular restriction on culturing conditions, and culturing may be carried out, for example, for about 12 to 48 hours while suitably controlling the pH and temperature to a pH range of 5 to 8 and a temperature range of 15 to 40°C under aerobic conditions.

[0030]

[II] Properties of L-Amino Acid Amide Hydrolases

The properties of L-amino acid amide hydrolase purified from *Corynebacterium glutamicum* strain ATCC 13286 among the aforementioned microbes will next be explained.

[0031]

The L-amino acid amide hydrolase at least has activity to produce L-alanine by hydrolyzing L-alanine amide, activity to produce L-alanyl-L-glutamine by using L-alanine amide and L-glutamine as substrates, and activity to produce L-alanyl-L-asparagine by using L-alanine amide and L-asparagine as substrates.

[0032]

The action of this L-amino acid amide hydrolase consists of hydrolyzing one molecule of L-alanine amide to produce one molecule of L-alanine and one molecule of ammonia, produce 1 molecule of L-alanyl-L-glutamine and 1 molecule of ammonia from one molecule of L-alanine amide and 1 molecule of L-glutamine, and produce one

molecule of L-alanyl-L-asparagine and one molecule of ammonia from 1 molecule of L-alanine amide and one molecule of L-asparagine.

[0033]

The optimum pH is in the vicinity of 6.0 to 10.0, and the optimum temperature is in the vicinity of 30°C to 50°C. The molecular weight of the subunit is calculated to be 42,000 to 46,000 as determined by SDS-polyacrylamide gel electrophoresis.

[0034]

[III] Dipeptide Producing Method

The dipeptide producing method of the present invention produces a dipeptide from an L-amino acid amide and an L-amino acid using a culture of a microbe having the ability to produce a dipeptide from an L-amino acid amide and an L-amino acid, microbial cells isolated from the culture, or a treated microbial product of the microbe.

[0035]

The L-amino acid amide hydrolase produced by the aforementioned microbe has activity to produce an L-amino acid by hydrolyzing an L-amino acid amide, and activity to produce a dipeptide by using an L-amino acid amide and an L-amino acid as substrates.

[0036]

Fig. 1 is a flow chart of the dipeptide producing method of the present invention.

A microbe having the ability to produce a dipeptide from an L-amino acid amide and an L-amino acid is first cultured in a medium, and the L-amino acid amide hydrolase is allowed to be produced and

accumulated in the medium and/or cells (Step S1).

Purified L-amino acid amide hydrolase is then produced by recovering and purifying the L-amino acid amide hydrolase (Step S2).

Subsequently, an L-amino acid amide and an L-amino acid are added to the purified L-amino acid amide hydrolase produced at Step S2 or to the medium in which the L-amino acid amide hydrolase has accumulated at Step S1 and the reaction is allowed to proceed to produce a dipeptide in large quantity (Step S3).

[0037]

As the method by which the L-amino acid amide hydrolase produced by the aforementioned microbes is allowed to act on the L-amino acid amide and L-amino acid, the substrates may be added directly to the culture liquid while culturing the aforementioned microbes, or microbial cells may be separated from the microbial culture by centrifugation and so forth, followed by either re-suspending in buffer either directly or after washing, and then adding an L-amino acid amide and an L-amino acid and allowing them to react. Alternatively, microbial cells can be used that have been immobilized by a known method using polyacrylamide gel, carrageenan or alginic acid gel.

[0038]

In addition, crushed microbial cells, acetone-treated microbial cells or freeze-dried microbial cells may be used as the treated microbial cell product. Methods such as ultrasonic crushing, French press crushing or glass bead crushing can be used for crushing microbial cells, while methods using egg white lysozyme, peptidase

treatment or a suitable combination thereof are used in the case of lysing microbial cells.

[0039]

Moreover, a L-amino acid amide hydrolase may be recovered from the treated microbial cell product and used as a crude enzyme liquid, or the enzyme may be purified before use as necessary. Ordinary enzyme purification methods can be used for purifying the enzyme obtained from a culture. More specifically, microbial cells are collected by centrifugation and so forth, the cells are then crushed by mechanical methods such as ultrasonic treatment, glass beads or a dynomill, and solid materials such as cell fragments are removed by centrifugation to obtain crude enzyme followed by purification of the aforementioned L-amino acid amide hydrolase by performing ultracentrifugation fractionation, salting out, organic solvent precipitation, ion exchange chromatography, adsorption chromatography, gel filtration chromatography, hydrophobic chromatography and so forth.

[0040]

Namely, in the case of a fraction having activity to produce a dipeptide from an L-amino acid amide and an L-amino acid, the whole enzyme and enzyme-containing substance can be used. Here, an "enzyme-containing substance" refers to that which contains the enzyme, and more specifically includes a culture, cultured microbial cells, washed microbial cells or processed microbial cells in which the cells have been crushed or lysed, a crude enzyme, purified enzyme and

so on.

[0041]

It should be noted that when the microbe used is a bacterium, since bacteria decompose dipeptides that have been produced simultaneous to their production, it may be preferable to produce the dipeptide using a treated microbial product, crude enzyme liquid, or purified enzyme rather than allowing the dipeptide production reaction to proceed while culturing the microbe.

[0042]

The amount of enzyme or enzyme-containing substance used may be enough if it is an amount in which the target effect is demonstrated (effective amount). This effective amount can be easily determined through simple, preliminary experimentation by a person with ordinary skill in the art; for example, in the case of using washed cells, the amount used is 1 to 500 g/l of reaction liquid.

[0043]

Any L-amino acid amide can be used as the L-amino acid amide so far as it can be hydrolyzed at the substrate specificity of the L-amino acid amide hydrolase, and examples of such include not only L-amino acid amides corresponding to naturally-occurring amino acids, but also L-amino acid amides corresponding to non-naturally-occurring amino acids or their derivatives. In addition, the L-amino acid amide hydrolase used in the present invention can also use racemic amino acid amides that can be synthesized inexpensively using the Strecker method since it imparts L-amino acids by asymmetrically hydrolyzing

racemic amino acid amides. In the present invention, L-alanine amide is used particularly preferably.

[0044]

There are no particular restrictions on the L-amino acid and a known one can be used so far as it forms a dipeptide with an L-amino acid amide at the substrate specificity of the L-amino acid amide hydrolase. In the present invention, L-glutamine or L-asparagine is used particularly preferably.

[0045]

Each concentration of the L-amino acid amide and L-amino acid used as starting materials is 1 mM to 10 M, and preferably 0.05 M to 2 M. However, there are cases in which it is preferable to add an equimolar amount or more of L-amino acid with respect to the amount of L-amino acid ester. In addition, in the case where a high concentration of substrate inhibits the reaction, it can be adjusted to a concentration that does not cause inhibition and then successively added during the reaction.

[0046]

The reaction temperature is 3 to 70°C, and preferably 5 to 50°C, while the reaction pH is 2 to 12, and preferably 3 to 11. By carrying out the reaction in this manner for about 2 to 48 hours, a dipeptide is produced and accumulates in the reaction mixture. Since the dipeptide production reaction is an equilibrium reaction, the reaction may be allowed to proceed after separating the dipeptide and ammonia produced to ensure efficient production.

[0047]

[Examples]

Hereinafter, the present invention will be described in detail by referring to the examples described below. However, the present invention is not limited to these examples. Note that in the examples, quantitative determination of L-alanine, L-alanyl-L-glutamine or L-alanyl-L-asparagine was carried out by a method using high performance liquid chromatography (column: Inertsil ODS-2 (GL Science), eluate: aqueous phosphate solution (pH 2.1), 2.5 mM sodium 1-octane sulfonate/methanol = 100/1), flow rate: 1.0 mL/min, detection: 210 nm).

[0048]

Example 1 Production of L-Alanyl-L-Asparagine

50 mL of a medium (pH 7.0) containing 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) glycerol, 0.5% (w/v) sodium chloride, and 0.5% (w/v) L-alanine amide hydrochloride were transferred to a 500 mL Sakaguchi flask and sterilized for 20 minutes at 120°C. One loopful of microbial cells of each of the microbes shown in Table 1, which had been cultured for 24 hours at 30°C on a slant medium containing 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) glycerol, 0.5% (w/v) sodium chloride, 0.5% (w/v) L-alanine amide hydrochloride, and 2% (w/v) agar (pH 7.0), was inoculated and cultured by shake culturing for 20 hours at 30°C and 120 strokes/minute. Following culturing, the microbial cells were separated by centrifugation, washed twice with physiological saline at a volume equal to the culture

liquid, and again separated by centrifugation followed by collecting the microbial cells and suspending in 0.2 M Tris-HCl buffer (pH 9.0) to a volume of 10 mL. 1 mL of the microbial suspension was added to 4 mL of the aforementioned buffer containing 62.5 mM L-alanine amide and 250 mM L-asparagine, and after bringing to a total volume of 5 mL, the mixture was allowed to react for 24 hours at 30°C. A section to which microbial cells were not added was established as a control experiment. Those results are shown in Table 1.

[0049]

[Table 1]

Microbe	Product L-Ala-L-Asn (mM)
Bacillus megateirum FERM P-18421	0.4
Corynebacterium glutamicum ATCC 13286	1.8
Erwinia carotovora FERM P-18420	0.5
Rhodococcus rhodochrous ATCC 19149	1.0
Chryseobacterium meningosepticum ATCC 13253	0.1
Micrococcus luteus ATCC 9341	0.1
Pseudomonas saccharophilia ATCC 9114	0.1
Cryptococcus albidus var. albidus IFO 610	1.8
Trichosporon gracile ATCC 24660	2.5
Rhodosporidium diobovatum ATCC 22264	2.7
Sporobolomyces salmonicolor IFO 1038	1.5
Tremela foliacea IFO 9297	3.3
Torulaspora delbrueckii IFO 1083	2.9

Sterigmatomyces elviae IFO 1843	0.1
Rhodotorula ingeniosa ATCC 22993	0.1
No addition of microbial cells	Below detection limit

L-Ala-L-Asn: L-alanyl-L-asparagine

[0050]

Example 2 Purification of L-Alanine Amide Hydrolase from *Corynebacterium glutamicum* strain ATCC 13286

Enzyme titer was measured in the manner described below. 200 μmol of Tris-HCl buffer (pH 9.0), 50 μmol of L-alanine amide, and a suitable amount of enzyme liquid were added and mixed to bring to a final volume of 1 ml, and after allowing to react for 60 minutes at 30°C, the reaction was stopped by adding 4 ml of aqueous phosphoric acid (pH 2.1). The L-alanine that was produced was quantified by high-performance liquid chromatography. The amount of enzyme that produces 1 μmol of L-alanine in 1 minute was defined as 1 unit of enzyme.

[0051]

8 L of *Corynebacterium glutamicum* strain ATCC 13286 was cultured in the same manner as Example 1, and the microbial cells were collected by centrifugation. The following procedure was carried out either on ice or at 4°C. After washing the microbial cells with 50 mM potassium phosphate buffer (pH 7.0), the cells were subjected to crushing treatment for about 10 minutes using glass beads having a diameter of 0.1 mm. The glass beads and crushed cell liquid were

then separated, and the crushed cell fragments were removed by centrifugation for 30 minutes at 20,000 x gravity (g) to obtain a cell-free extract. Moreover, the insoluble fraction was removed by ultracentrifugation for 60 minutes at 200,000 x g to obtain a soluble fraction in the form of the supernatant. Ammonium sulfate was added to the resulting soluble fraction to 60% saturation, and the precipitate was recovered by centrifugation for 30 minutes at 20,000 x g. The resulting precipitate was dissolved in a small amount of 50 mM potassium phosphate buffer (pH 7.0) and then dialyzed against 50 mM potassium phosphate buffer (pH 7.0). This enzyme liquid was then applied to a Q-Sepharose HP column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0), and the enzyme was eluted over a linear concentration gradient of 50 mM potassium phosphate buffer (pH 7.0) containing 0 to 1.0 M sodium chloride. The active fraction was collected and applied to a Superdex 200 pg column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0), and the enzyme was then eluted with the same buffer. The active fraction was collected and dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 0.5 M ammonium sulfate, and then applied to a Phenyl-Sepharose HP column pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.5 M ammonium sulfate. The enzyme was then eluted over a linear concentration gradient of 20 mM potassium phosphate buffer (pH 7.0) containing 0.5 to 0 M ammonium sulfate. The active fraction was collected and dialyzed against 50 mM potassium phosphate buffer (pH 7.0), and this was then applied to a

MonoQ column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0), after which enzyme was eluted over a linear concentration gradient of 50 mM potassium phosphate buffer (pH 7.0) containing 0 to 1.0 M sodium chloride. The L-alanine amide hydrolase was uniformly purified on the basis of electrophoresis in this manner. The total amount of protein and the specific activity in each purification step are shown in Table 2.

[0052]

[Table 2]

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)
Cell-free extract	80	2000	0.040
Soluble fraction	71	1690	0.042
Ammonium sulfate fraction	79	1080	0.073
Q-Sepharose HP	55	379	0.149
Supeprdex200pg	21	151	0.135
Phenyl-Sepharose HP	12.5	6.60	1.897
MonoQ	2.4	0.24	9.841

[0053]

Example 3 Evaluation of Molecular Weight of L-Alanine Hydrolase

The equivalent of 0.5 μg of the purified enzyme standard obtained according to the method of Example 2 was applied to polyacrylamide electrophoresis. 0.3% (w/v) Tris, 1.44% (w/v) glycine, and 0.1% (w/v) sodium lauryl sulfate were used for the electrophoresis buffer, concentration gradient gel (Multi-gel 10-20, produced by Daiichi

Pure Chemicals Co., Ltd.) having a gel concentration of 10 to 20% was used for the polyacrylamide gel, and precision per-stained standards (produced by Bio-Rad Laboratories, Inc.) were used for the molecular weight markers. Following completion of electrophoresis, the gel was stained with Coomassie-Brilliant Blue R-250, and a uniform band was detected at the position calculated to represent a molecular weight of 42,000 to 46,000.

[0054]

Example 4 Evaluation of Optimum pH of L-Alanine Amide Hydrolase

L-alanine amide was hydrolyzed using the L-alanine amide hydrolase uniformly purified in Example 2, and reaction pH was evaluated as described below for the reaction that produces L-alanine. 200 μmol of sodium acetate buffer (pH 3.0 to 6.0), potassium phosphate buffer (pH 6.0 to 8.0), Tris-HCl buffer (pH 7.0 to 9.0), sodium carbonate buffer (pH 8.0 to 10.0), and glycine-sodium hydroxide buffer as buffer solution, 50 μmol of L-alanine amide, and a suitable amount of enzyme liquid were added and mixed to a final volume of 1 ml followed by evaluating enzyme activity by reacting for 60 minutes at 30°C. The results based on a value of 100% for the activity when using Tris-HCl buffer (pH 8.0) are shown in Fig. 1.

[0055]

Example 5 Evaluation of Reaction Temperature of L-Alanine Amide Hydrolase

L-alanine amide was hydrolyzed using the L-alanine amide hydrolase uniformly purified in Example 2, and reaction temperature

was evaluated as described below for the reaction that produces L-alanine. 200 μmol of tris HCl buffer, 50 μmol of L-alanine amide, and a suitable amount of enzyme liquid were added and mixed to a final volume of 1 ml followed by evaluating enzyme activity by reacting for 60 minutes at 25, 30, 40, 50, and 60°C. The results based on a value of 100% for the activity when reacting at reaction temperature of 40°C are shown in Fig. 2.

[0056]

Example 6 Production of L-Alanyl-L-Asparagine and L-Alanyl-L-Glutamine

L-alanyl-L-asparagine or L-alanyl-L-glutamine was produced by allowing the L-alanine amide hydrolase uniformly purified in Example 2 to act on L-alanine amide and L-asparagine or on L-alanine amide and L-glutamine. When obtaining L-alanyl-L-asparagine, 200 μmol of Tris-HCl buffer (pH 9.0), 50 μmol of L-alanine amide, 150 μmol of L-asparagine, and enzyme liquid of L-alanine amide hydrolase activity of 0.08 unit were added and mixed to a final volume of 1 ml. When obtaining L-alanyl-L-glutamine, with the exception of using 150 μmol of L-glutamine instead of 150 μmol of L-asparagine, mixing was carried out under the same conditions as in the case of obtaining L-alanyl-L-asparagine. Sections in which only one substrate was added or a section in which enzyme was not added were established as control experiments. The reaction was allowed to proceed for 10 hours at a reaction temperature of 30°C, and the results of quantifying the target products are shown in Table 3.

[0057]

[Table 3]

Substrate		Enzyme added	Product	Product concentration (mM)
L-alanine amide	L-asparagine	Yes	L-alanyl-L-asparagine	8.4
L-alanine amide	-	Yes	L-alanyl-L-asparagine	Below detection limit
-	L-asparagine	Yes	L-alanyl-L-asparagine	Below detection limit
L-alanine amide	L-asparagine	No	L-alanyl-L-asparagine	Below detection limit
L-alanine amide	L-glutamine	Yes	L-alanyl-L-glutamine	7.7
L-alanine amide	-	Yes	L-alanyl-L-glutamine	Below detection limit
-	L-glutamine	Yes	L-alanyl-L-glutamine	Below detection limit
L-alanine amide	L-glutamine	No	L-alanyl-L-glutamine	Below detection limit

[0058]

[Effects due to the Invention]

According to the dipeptide producing method of the present invention, a dipeptide can be produced using L-amino acid amide and L-amino acid which can be acquired comparatively inexpensively without going through a complex synthesis method, making it possible to reduce the production cost of dipeptides useful as pharmaceutical materials, functional foods, and the like.

[0059]

In addition, the L-amino acid amide hydrolase of the present invention can be preferably used in the dipeptide producing method of the present invention.

[Brief Description of the Drawings]

[Fig. 1]

Fig. 1 is a flow chart showing the steps of the dipeptide producing method of the present invention.

[Fig. 2]

Fig. 2 depicts the optimum pH curve of L-amino acid amide hydrolase derived from *Corynebacterium glutamicum* strain ATCC 13286.

[Fig. 3]

Fig. 3 depicts the optimum temperature curve of the L-amino acid amide hydrolase derived from the *Corynebacterium glutamicum* strain ATCC 13286.

[Type of Document] Abstract

[Abstract]

[Object] To provide a method for producing dipeptide using inexpensively acquirable starting materials by an industrially advantageous and simple pathway.

[Means] Dipeptide is produced from L-amino acid amide and L-amino acid using a culture of microbes having the ability to produce a dipeptide from an L-amino acid amide and an L-amino acid, using microbial cells isolated from the culture, or a treated microbial cell product of the microbe.

[Selected Figure] None

FIG.1

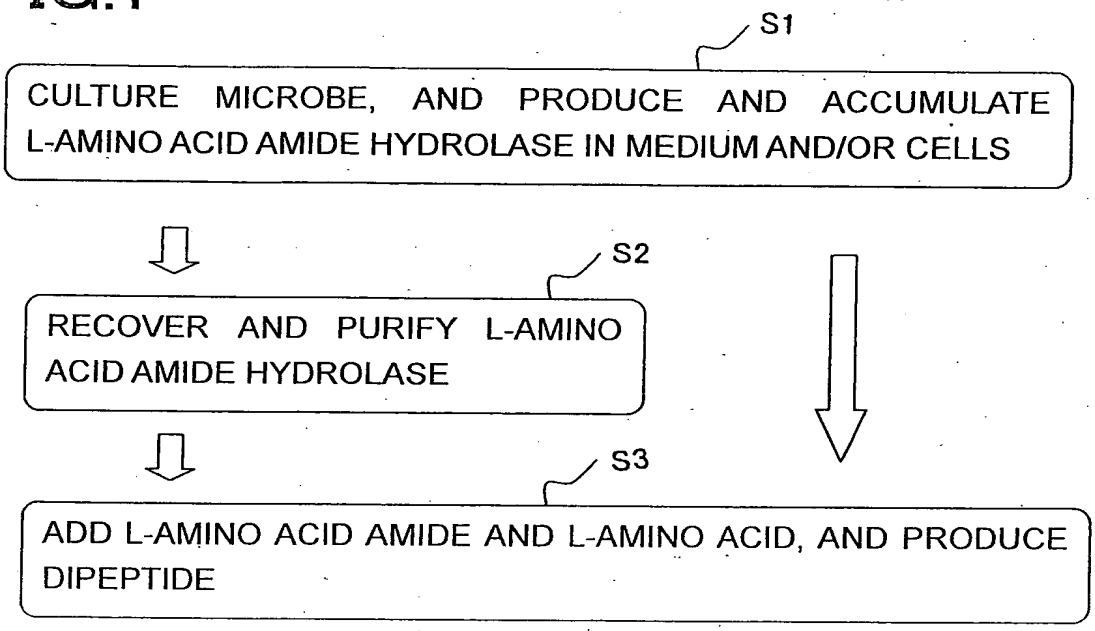

S1
CULTURE MICROBE, AND PRODUCE AND ACCUMULATE L-AMINO ACID AMIDE HYDROLASE IN MEDIUM AND/OR CELLS
S2
RECOVER AND PURIFY L-AMINO ACID AMIDE HYDROLASE
S3
ADD L-AMINO ACID AMIDE AND L-AMINO ACID, AND PRODUCE DIPEPTIDE

FIG.2

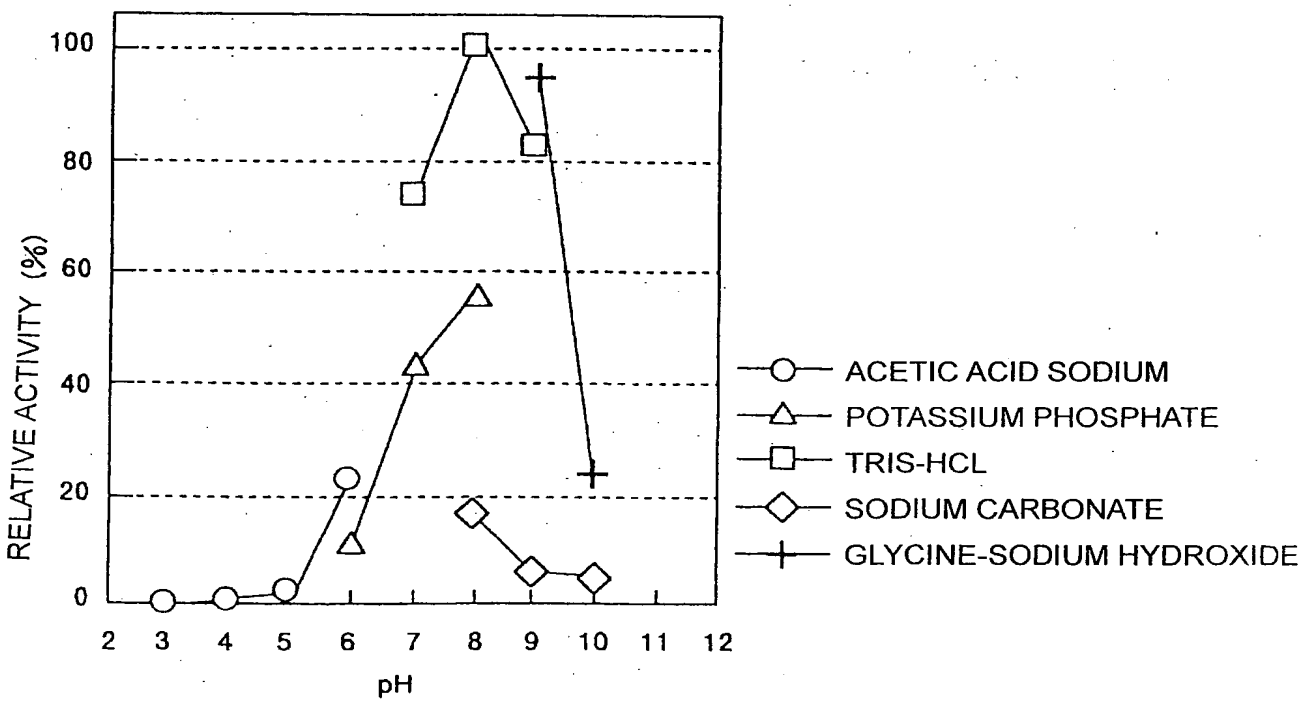
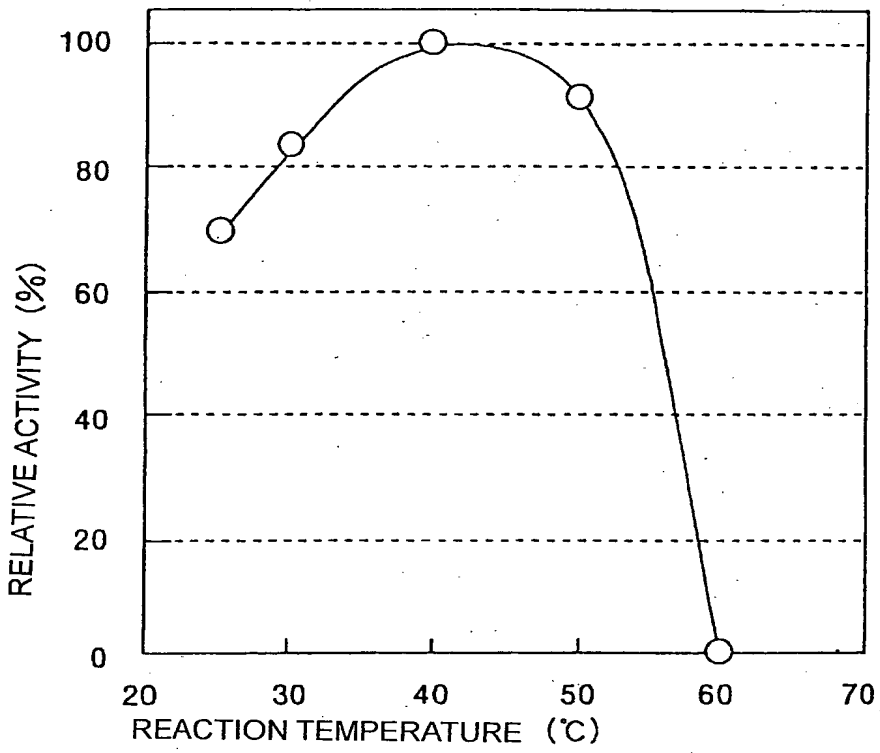

RELATIVE ACTIVITY (%)
100
80
60
40
20
0
2
3
4
5
6
7
8
9
10
11
12
pH
ACETIC ACID SODIUM
POTASSIUM PHOSPHATE
TRIS-HCL
SODIUM CARBONATE
GLYCINE-SODIUM HYDROXIDE

FIG.3


100
80
60
40
20
0
RELATIVE ACTIVITY (%)
20
30
40
50
60
70
REACTION TEMPERATURE (℃)